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Prodrugs of butyric acid. Novel derivatives possessing increased aqueous solubility and potential for treating cancer and blood diseases

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Abstract – The synthesis and biological activities of acidic, basic and neutral types of butyric acid (BA) prodrugs possessing increased aqueous solubility are described. The compounds are butyroyloxyalkyl derivatives of carboxylic acids, which possess functionalities suitable for aqueous solubilization. The anticancer activity of the prodrugs in vitro was evaluated by examining their effect on the growth of human colon, breast and pancreatic carcinoma cell lines, and their solubility in aqueous media was determined. The most promising compounds, with respect to activity and solubility, were found to be the butyroyloxymethyl esters of glutaric **2a** and nicotinic acids **4a** and phosphoric acid as its diethyl ester **10a**, which displayed IC_{50} values of 100 μM or lower. These prodrugs are expected to release formaldehyde upon metabolic hydrolysis. The corresponding butyroyloxyethyl esters (**2b**, **4b** and **10b**) that release acetaldehyde upon metabolism were significantly less potent. A similar correlation was observed for growth inhibition of the human prostate carcinoma cell lines PC-3 and LnCap and for induction of differentiation and apoptosis in the human myeloid leukemia cell line HL-60. The higher biological activity of the formaldehyde-releasing prodrugs **2a** and **10a** was further confirmed when induction of hemoglobin (Hb) synthesis in the human erythroleukemic cell line K562 was measured. Moreover, a therapeutic index (IC_{50}/ED_{50}) of ca. 5 was observed. The acute i.p. toxicity LD_{50} in mice for **2a**, **2b**, **10a** and **10b** was similar and in the range of 400–600 mg kg^{-1}. The results obtained support the potential use of the butyric acid prodrugs for the treatment of neoplastic diseases and β-globin disorders. © 2001 Éditions scientifiques et médicales Elsevier SAS

prodrugs / butyric acid / anticancer / water solubility

1. Introduction

Previous publications from our laboratories have described the synthesis and anticancer activity of butyric acid (BA) prodrugs, that have been shown to modulate gene expression, induce histone hyperacetylation, differentiation, and apoptosis of cancer cells [1–5]. Pivaloyloxymethyl butyrate (**AN-9**), the best-studied prodrug, affects cancer cells about 100-fold faster and at about 10-fold lower concentration than does BA [1, 2]. Moreover, **AN-9** penetrates 100-fold faster than BA into cancer cells in vitro [6].

Esterification of BA improves its permeability across cell membranes and enables efficient delivery of BA to a subcellular target. **AN-9** was shown to inhibit the proliferation of a variety of cancer cell lines [1, 2] as well as primary human tumors, including colorectal, breast, lung, ovarian, renal cell and urinary bladder [7]. The response to **AN-9** was dose-dependent and was significantly greater than that of BA. **AN-9** displayed low toxicity in mice and was effective in prolonging survival of mice bearing melanoma, lung carcinoma and monocytic leukemia

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[1, 2, 8]. It induces transient hyperacetylation of histones leading to relaxation of the chromatin structure and allowing access to transcription factors [3, 4, 9]. This activity is likely to be an important mechanism by which the prodrugs exert their effect on gene modulation. **AN-9** modulates the expression of the early regulatory genes, c-myc and c-jun, and the tumor suppressor gene RB as well as the anti-apoptotic gene bcl-2 in WEHI and HL-60 cells [3, 5, 10]. A synergistic effect of **AN-9** and daunorubicin was observed in murine monocytic leukemia cells resulting in markedly prolonged survival of mice with monocytic leukemia [8]. **AN-9**, formulated in lipid emulsion, labeled – PIVANEX®, in a phase I clinical study displayed low toxicity and was reported to have an estimated MTD of 2.69 g m^{-2} day^{-1} [11]. It is presently in Phase II clinical studies with non-small-cell lung carcinoma.

Another important application of BA prodrugs is in the treatment of β-globin disorders [12–16]. BA-prodrugs have been shown to increase hemoglobin (Hb) expression in several model systems. Among them, butylidene dibutyrate (**AN-10**) was found to be the most effective [17]. In humans, Hb production is characterized by two major 'switches' that involve the production of different β-type chains at different stages of development. The production of embryonic Hb switches into fetal hemoglobin (HbF) after the first two months of gestation and then again into adult hemoglobin before birth (HbA). In adult life, the major Hb is HbA, while HbF comprises <1% of the total Hb content. Inherited blood disorders, known as β-hemoglobinopathies, are characterized by reduced, or lack of, production of adult-type β-chains in β-thalassemia or in the formation of structurally abnormal β-globin chain in sickle cell anemia (SCA). In both cases, an increase in HbF production ameliorates the clinical symptoms of the underlying disease. Although much is known about the biochemical and molecular basis of these inherited blood diseases in man, similar progress in the development of suitable therapies has not been made [13].

In clinical trials conducted for the treatment of β-thalassemia and SCA, BA salts were reported to elevate expression of HbF [14, 16], thereby favorably modifying the disease symptoms. BA prodrugs, which possess low toxicity, may provide significant advantages for treatment of β-thalassemia and SCA, as compared to the cytotoxic agent hydroxyurea currently approved for the chronic treatment of SCA patients [18].

The design of the **AN-9** and **AN-10**, as well as other early analogs [1], resulted in highly lipophilic derivatives better able to cross cell membranes than BA [6]. However, they are virtually devoid of water solubility, requiring non-aqueous media for their clinical formulation. This report describes the synthesis and biological activity of novel types of BA prodrugs that may be more readily formulated in aqueous media, retain high anticancer activity and augment production of HbF.

2. Chemistry

In view of our earlier successful experience with acyloxyalkyl ester prodrugs of BA [1–6], analogous derivatives possessing water solubilizing functional groups were designed. These acetal diester prodrugs (**V**) are known to release equimolar amounts of the acidic drug (**I**), an aldehyde (R^1CHO, **VI**) and a second acid (R^2COOH, **IV**) upon metabolic breakdown [19]. The latter is the acidic component of the 'carrier'. The selection of 'carrier' moieties that upon metabolic cleavage would release fragments possessing minimal toxicity was of major importance in the design of the new prodrugs.

For this purpose, the acidic 'carriers' chosen were natural compounds found in the human body such as *p*-amino-benzoic acid (PABA) (LD_{50} po in rats >6 g kg^{-1}) [20a], nicotinic acid (LD_{50} sc in rats 5 g kg^{-1}) [20b], and phosphoric acid as its diethyl ester. Derivatives of other acids expected to possess low toxicity, such as glutaric acid (found in green sugar beets [20c], LD_{50} po in mice 6 g kg^{-1}, [21]), 2-(2-methoxyethoxy)acetic and [2-(2-methoxyethoxy)ethoxy]acetic acids were also prepared. Although no prohibitive toxicity has been reported with the use of prodrugs that release formaldehyde such as Pivampicillin [20d] or PIVANEX®, for comparative purposes acyloxymethyl derivatives that release formaldehyde ($R^1 = H$) and the corresponding 1-acyloxyethyl derivatives that release acetaldehyde ($R^1 = Me$) were prepared (*figure 1*).

The novel BA prodrugs prepared are of acidic, basic and neutral types.

2.1. Acidic derivatives

Chloromethyl butyrate, **1a**, was prepared from butyryl chloride/paraformaldehyde/$ZnCl_2$ at 80 °C, as de-

scribed for chloromethyl pivalate [22], whereas 1-chloroethyl butyrate, **1b**, was prepared from butyryl chloride/acetaldehyde/$ZnCl_2$ at 0 °C [23]. Reaction of **1a** and **1b** with glutaric acid gave **2a** and **2b** accompanied by the respective neutral side products **3a** and **3b** stemming from esterification at both ends of the glutaric acid (*figure 2*).

2.2. Basic derivatives

Reaction of nicotinic acid with **1a** or **1b**, led to the respective nicotinic acid esters **4a** and **4b** (*figure 2*). Reaction of PABA with **1a** gave **5**. The basic prodrugs **4a**, **4b** and **5** were converted into their respective acid addition salts with inorganic acids.

2.3. Neutral derivatives

Since polyalkoxyacetic acid derivatives have been reported to provide water solubility to their esters [24], prodrugs **8** and **9** were prepared from chloromethyl butyrate, **1a**, and 2-(2-methoxyethoxy)acetic and [2-(2-methoxyethoxy)ethoxy]acetic acids, respectively (*figure 2*). Prodrugs **10a** and **10b**, which upon hydrolysis are expected to give BA, the corresponding aldehydes, ethanol and phosphoric acid as final metabolites, were prepared by esterification of **1a** and **1b** with diethyl phosphate [25]. Other neutral, water insoluble derivatives, **3a**, **3b**, **7a** and **7b**, were also included in the comparative activity studies. The latter two, derived from PABA, were prepared from amide **6**.

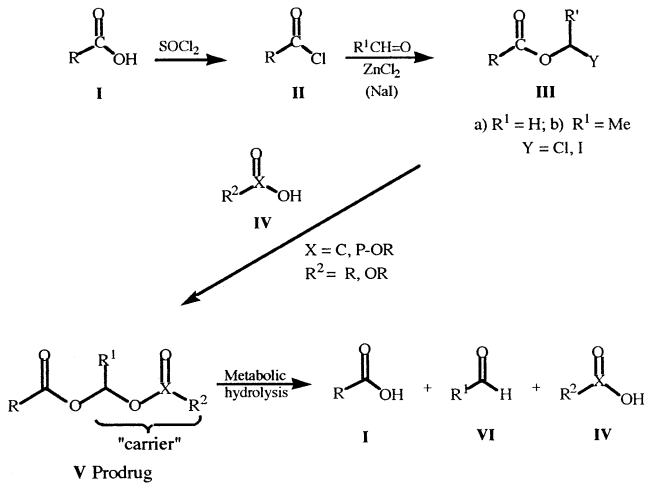

Figure 1. General scheme for synthesis and hydrolysis of butyric acid (BA) prodrugs.

The water solubilities of the prodrugs were determined as described in the legend of *table I* and were compared to those of **AN-9** and **AN-10**. The latter exhibited poor solubility (<1 mg mL^{-1}), whereas the acidic prodrugs **2a** and **2b** were found to be slightly soluble per se, their alkali metal or lysine salts were highly soluble. Basic prodrugs **4a** and **5** were poorly soluble, but their acid addition salts were highly soluble. Neutral prodrugs **8** and **9** derived from methoxy-alkoxy-acetic acids, and **10a** and **10b** derived from diethyl phosphate, had significantly improved solubilities attributed to their high oxygen content. As expected, methylidene derivatives **2a** and **10a** were more soluble than the corresponding ethylidene ones **2b** and **10b** (*table I*).

3. Biology

3.1. Growth inhibition of human colon, breast and pancreatic cancer cell lines

Growth inhibition activity on the colon HT-29, breast MCF-7 and pancreatic $MIAPaCa_2$ carcinoma cell lines was determined by the sulforhodamine blue (SRB) assay (*table II*). Similar tests have been used by the NCI for in vitro screening of potential anticancer drugs [27]. Among the group of acidic prodrugs the most active one was mono-butyroyloxymethyl glutarate **2a**. In the group of basic prodrugs the nicotinic acid derivative **4a** displayed similar activity to **2a**. The PABA derivative **5** was less active than **4a**. In the group of the neutral prodrugs, compounds **3a**, **8**, **9** and **10a** had antiproliferative activity comparable to that of **2a**.

Although on a molar basis, **2a** releases 1 equiv. of BA upon hydrolysis, whereas **3a** releases 2 equiv. of the acid, both compounds were found to display similar activity. This indicates that the total number of equivalents of BA present in the molecule is not the determining factor in the potency of prodrug. The lack of correlation between the number of BA equivalents released and the activity of the compounds has been observed previously, e.g. **AN-9** vs. **AN-10** [1]. The lower activity of **7a** as compared to **5** could be attributed to slow intracellular amide hydrolysis. For practical purposes, **2a** and **5** have the advantage of lower molecular weight and higher aqueous solubility than **3a** and **7a**, respectively.

It should be noted that exposure to the nicotinic acid derivatives **4a** and **4b** caused a potent, albeit transient

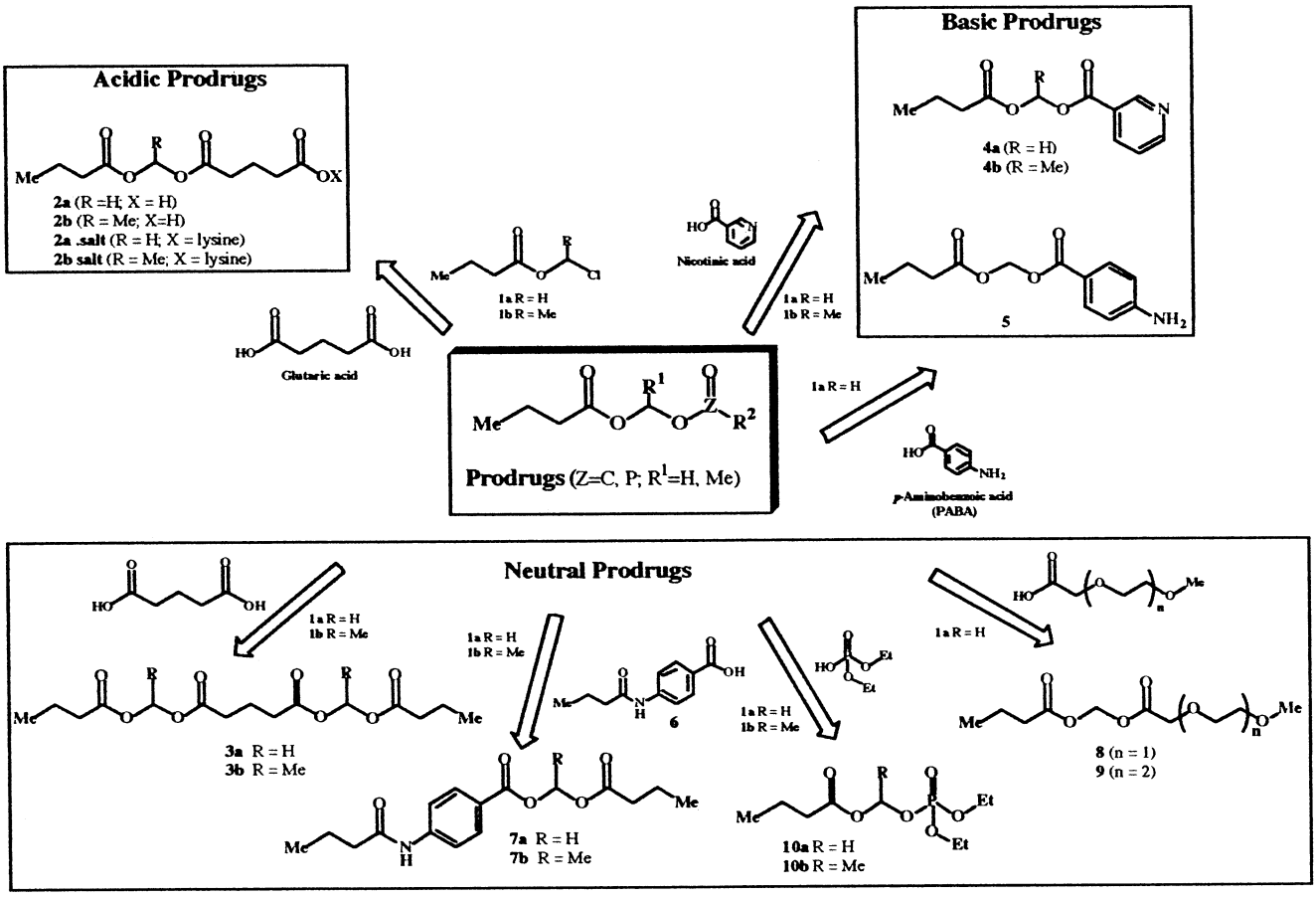

Figure 2. Acidic, basic and neutral prodrugs of butyric acid (BA).

peripheral skin vasodilation of the persons handling these compounds and therefore they were excluded from further evaluation. This effect is similar to that reported for other nicotinic acid esters and amides, such as Nicametate [20c].

All the BA prodrugs that release formaldehyde upon hydrolysis were found to be considerably more active than the corresponding analogs that release acetaldehyde. Since formaldehyde is a more reactive species, it may contribute to the higher antiproliferative activity observed in vitro for the former compounds (*table II*). It is reasonable to assume that the released formaldehyde may also contribute to the in vivo toxicity in mice. To clear up this assumption, compounds **2a**, **2b**, **10a**, and **10b** were evaluated for in vivo toxicity in mice. However, the acute toxicity values of a single i.p. dose (LD_{50} in Balb-c mice), of all four compounds were similar ranging between 400–600 mg kg^{-1}. It can be concluded that intracellularly released formaldehyde affects cancer cells specifically, without significantly contributing to the overall toxicity. Therefore, the formaldehyde releasing BA-prodrugs, as anticancer agents, have an advantage over the acetaldehyde-releasing ones.

Although **2a** and **2b** displayed satisfactory aqueous solubility, they were converted to their lysine salts to further increase their solubility profile. The salts were indeed more soluble and had lower in vivo toxicity than the corresponding free acids, (LD_{50}>1000 mg kg^{-1}), but were found to be less potent inhibitors of human prostate cancer cells than the respective free acids (*table III*).

In view of the water solubility and activity profiles of the acidic **2a** and neutral **10a** prodrugs (*tables I* and *II*), subsequent biological evaluations were expanded for these compounds in parallel with their analogs **2b** and **10b**, as described below.

3.2. Inhibition of proliferation of human prostate cell lines

The inhibition of proliferation of the hormone-dependent (LnCap) and hormone-independent (PC-3) human prostate carcinoma cell lines, was determined using an XTT assay [28]. The results obtained confirm the higher antiproliferative activity (at least 7-fold) of the methylidene derivatives **2a** and **10a** as compared to their respective ethylidene analogs **2b** and **10b** (*table III*).

3.3. Induction of differentiation and apoptosis, and inhibition of proliferation of human leukemia cells

The differentiation activity of prodrugs **2a**, **2b**, **10a** and **10b** on the myeloid leukemia cell line HL-60 was determined by nitro blue tetrazolium (NBT) reduction activity, and inhibition of proliferation was evaluated by ^{3}[H]-thymidine incorporation [1, 2]. Apoptosis induction was evaluated by FITC-conjugated annexin and analyzed by flow cytometry [29]. The results demonstrate that in addition to inhibiting proliferation, the formaldehyde-releasing prodrugs **2a** and **10a**, also displayed higher potency (at least 3-fold), than **2b** and **10b** in promoting differentiation and apoptosis (*table IV*).

Table I. Aqueous solubility of the prodrugs.[a]

Prodrug	Solubility mg mL^{-1}
AN-9	<1
AN-10	<1
2a	22
2b	6
3a	<1
3b	<1
4a	3.5
4b	<1
5	<1
7a	<1
7b	<1
8	>40
9	>40
10a	18.5
10b	3

[a] Solubility in 25 mM phosphate buffer, pH 7, at 25 °C was measured by the shake flask method [26], suspending 40 mg of the prodrug in 1 mL of buffer and increasing the volume of buffer, up to 40 mL, until complete dissolution occurred. Compounds that did not dissolve at this concentration are indicated as possessing solubility of <1 mg mL^{-1}.

Table II. Proliferation inhibition of human colon-HT-29, breast-MCF-7 and pancreatic-MIAPaCa$_2$ cancer cell lines by the acidic, basic and neutral BA prodrugs.[a]

Prodrug	IC_{50} μM[b]		
	HT-29	MCF-7	MIAPaCa$_2$
2a	98	110	32
2b	3060	1900	1900
3a	95	94	84
3b	>3000[c]	>3000	>3000
4a	100	126	58
4b	3100	2900	1500
5	190	390	220
7a	360	570	450
7b	1450	1540	1500
8	164	95	185
9	210	160	210
10a	100	95	91
10b	2420	2900	1270

[a] Cells at a density of 5×10^4 cells mL^{-1} were plated in 96-well plates and after 24 h the test drugs were added. The drugs, dissolved in DMSO, were diluted with growth media to give a final DMSO concentration in the well of 0.1%. Ten prodrug concentrations ranging from 4 to 0.008 mM were used in triplicate. The negative controls were untreated cells and positive controls were cells treated with 5 μM doxorubicin. After seven days of incubation the samples were stained with 0.4% SRB and read for absorbance at 570 nm.
[b] The correlation factors obtained for the above dose–response studies were >0.9.
[c] At concentration above 3 mM, **3b** could not be solubilized in the media used.

3.4. Induction of Hb synthesis

Induction of Hb synthesis is considered as a marker of differentiation activity in the erythroleukemic cell line, K562 [30]. Since salts of BA have been reported to elevate expression of Hb [14–16], the effect of the prodrugs on the induction of cellular Hb (ED_{50}) was evaluated by the HPLC method described in [31]. Parallel inhibition of proliferation of these cells (IC_{50}) was measured by ^{3}[H]-thymidine incorporation into cellular DNA, which took place at much higher doses (*table V*). While BA increased the production of Hb in the cells with an ED_{50} of 179±63 μM, prodrugs **2a** and **10a** displayed ED_{50} values of ca. 30 μM and IC_{50} above 100 μM, giving an overall IC_{50}/ED_{50} ratio of about 5. Compound **2b** had lower activity. It is important for treatment of chronic β-hemoglobinopathies, that the drugs should induce Hb synthesis with high potency and display low cytotoxicity, in particular toward Hb prod-

Table III. Growth inhibition of human prostate cancer cell lines.

Prodrug	IC_{50} μM[a]	
	PC-3	LnCap
2a	150 ± 22	89 ± 5
2b	1395 ± 420	650 ± 17
10a	128 ± 33	122 ± 7
10b	3249 ± 421	981 ± 123
2a lysine salt	nd[b]	569 ± 28
2b lysine salt	nd[b]	1081 ± 82

[a] IC_{50} values were determined by the XTT assay after 48 h of exposure to the prodrugs. The IC_{50} values represent the average and standard deviation of at least three independent experiments.
[b] not determined.

ucing cells. Prodrugs **2a** and **10a** meet these criteria. It is worth noting that to date all the agents that induced Hb accumulation in K562 cells were also effective in inducing HbF in vivo (both in experimental animals and patients) [15, 16]. Therefore, the results obtained in this experimental model suggest that **2a** and **10a** may have a therapeutic efficacy in patients with β-globin disorders (e.g. sickle cell anemia and β-thalassemia).

4. Conclusions

The chemical design of BA prodrugs led to modification of their physico–chemical properties so that a series of derivatives ranging from highly lipophilic to hydrophilic were obtained. The compounds possessing moderate water solubility in the range of 3–22 mg mL^{-1} were found most advantageous for drug formulation. In addition, other structural features of the compounds turned out to be important for their biological activities. A comparison of inhibition of human carcinoma cell lines by the prodrugs that release formaldehyde (compounds **2a**, **4a**, **7a** and **10a**) with their acetaldehyde-releasing counterparts (compounds **2b**, **4b**, **7b** and **10b**) showed that the former were significantly more active. A similar correlation was found for induction of differentiation and apoptosis in the human myeloid leukemia cell line HL-60 as well as for induction of Hb synthesis in the erythroleukemic cell line K562. We postulate that this difference is related to the higher reactivity of the released formaldehyde. Although formaldehyde-releasing prodrugs display higher in vitro activity compared to acetaldehyde releasing ones, both series of compounds displayed similar acute toxicity LD_{50} values in mice. The mechanism by which the aldehyde moiety of the prodrugs contributes to the anticancer activity in now under investigation.

Table IV. Induction of apoptosis and differentiation, and inhibition of proliferation of HL-60 cells.

Prodrug	Apoptosis ED_{50}, μM[a]	Proliferation IC_{50}, μM[b]	Differentiation ED_{50}, μM[c]
2a	77	72	81 ± 28
2b	220	205	150 ± 42
10a	70	50	58 ± 9.6
10b	230	192	218 ± 54

[a] The values were determined for cells stained with annexin and propidium iodide and subjected to FACS analysis.
[b] Proliferation was determined by ^{3}[H]-thymidine incorporation, as described in the experimental section. The data presented for apoptosis and proliferation were based on a representative single set of data. The linearity of the data is reflected by the correlation factor >0.95.
[c] The ED_{50} values for differentiation, measured by % NBT positive cells, were derived from linear regression of three dose–response experiments.

Table V. The effect of BA prodrugs on Hb synthesis and inhibition of proliferation in the human erythroleukemic cell line K562.[a]

Compound	ED_{50} μM	IC_{50} μM
2a	22 ± 7	164
2b	89 ± 15	601
10a	26 ± 0.3	119
10b	nd[b]	553

[a] Hb synthesis was determined by HPLC and proliferation inhibition was measured by ^{3}[H]-thymidine incorporation into K562 cells, as described in the experimental section. The linearity of the IC_{50} values obtained was high as is evident by the high correlation coefficient ≥ 0.90.
[b] not determined.

5. Experimental protocols

5.1. Chemistry

^{1}H-NMR and ^{13}C-NMR spectra were obtained on Bruker AC-200, DPX-300 and DMX-600 spectrometers. For $CDCl_3$ and acetone-d_6 solutions, chemical shifts are expressed in ppm downfield from Me_4Si used as internal standard; for D_2O solutions the HOD peak was taken as

δ 4.80 (^{1}H-spectra), or the peak of a small amount of added MeOH taken as δ 49.50 (^{13}C). For ^{31}P spectra an external standard of 85% H_3PO_4 was used. Multiplicities in the ^{13}C-NMR spectra were determined by off-resonance decoupling. Mass spectra were obtained on a Finnigan 4021 spectrometer operating in CI (chemical ionization), DCI (desorption chemical ionization), EI (electron impact) or HRMS (high-resolution) modes. Progress of the reactions was monitored by TLC on silica gel or alumina. Melting points were determined on a Fisher–Johns apparatus. Analyses indicated by the symbols of the elements were within ±0.4% of the theoretical values

5.1.1. Butyric acid chloromethyl ester ***1a***

The ester was prepared by a modification of the reported procedure [22]. A mixture of butyryl chloride (944.2 g, 8.86 mol), paraformaldehyde (265.9 g, 8.86 mol) and $ZnCl_2$ (cat.) were stirred at r.t. An exothermic reaction occurred after 10 min, whereby the temperature reached 75–80 °C. After 20–25 min, the temperature dropped and the reaction was heated at 75 °C for 3 h. The mixture was distilled at 40 Torr, whereby four fractions having the following boiling points (b.p.) were collected: a) 47–87 °C, b) 87–91 °C, c) 91–92 °C, d) >92 °C. The combined fractions c and d were redistilled to give the product as a colorless oil (769.4 g, 64% yield). ^{1}H-NMR ($CDCl_3$) δ 5.69 (s; 2H, OCH_2Cl); 2.36 (t; $J = 7.5$ Hz, 2H, CH_2CO); 1.68 (sextet; $J = 7.5$ Hz, 2H, CH_2Me); 0.97 (t; $J = 7.5$ Hz, 3H, *Me*). ^{13}C-NMR ($CDCl_3$) δ 171.39 (s; CO); 68.52 (t; OCH_2Cl); 35.78 (t; CH_2CO); 18.04 (t; $MeCH_2$); 13.44 (q; Me).

5.1.2. Butyric acid 1-chloroethyl ester ***1b*** *[23]*

Cooled acetaldehyde (190 mL, 3.3 mol) was added dropwise to a cooled (−20 °C, ice-salt bath), stirred mixture of butyryl chloride (320 g, 3 mol) and $ZnCl_2$ (ca. 0.3 g) under N_2 in the course of 0.5 h. At the end of the addition, the mixture was cooled for an additional 1 h. A ^{1}H-NMR spectrum taken at room temperature after 1 h showed almost pure product. The crude material was dissolved in petroleum ether, and was eluted through a silica column (silica gel 60 H, 6×8 cm) under suction. The eluted solution was filtered, evaporated, and the residue was vacuum distilled (water pump) at 89–105 °C, to give the product (330 g, 74% yield). ^{1}H-NMR ($CDCl_3$) δ 6.55 (q; $J = 6.0$ Hz, OC*H*O, 1H); 2.34 (t; $J = 7.5$ Hz, 2H, CH_2CO); 1.78 (d; $J = 6.0$ Hz, 1H, CH*Me*); 1.68 (sextet; $J = 7.5$ Hz, 2H, $MeCH_2$); 0.96 (t; $J = 7.5$ Hz, 3H, Me).

5.1.3. Diethyl phosphate [25]

NaOH (1N, 100 mL) was added dropwise to an ice-cold solution of diethyl chlorophosphate (17.3 g, 0.1 mol) in THF. After stirring the mixture for 0.5 h, the solvent was evaporated and the product obtained was a clear oily residue (95% yield), which was used without further purification. ^{1}H-NMR ($CDCl_3$) δ 9.92 (brs; 1H, OH); 4.12 (q; $J = 7.0$ Hz, 2H, CH_2Me); 4.09 (q; $J = 7.0$ Hz, 2H, CH_2Me); 1.34 (td; $J = 7.0$, 1.0 Hz, 6H, two Me). ^{13}C-NMR ($CDCl_3$) δ 63.63 (d; $J_{CP} = 5.5$ Hz, CH_2); 15.96 (d; $J_{CP} = 7.0$ Hz, Me).

5.1.4. 4-Butyrylamino-benzoic acid ***6***

Butyric anhydride (18 mL, 17.3 g, 1 equiv.) was added dropwise to an ice-cold solution of 4-amino-benzoic acid (15.12 g, 109 mmol) in dry pyridine (100 mL) under N_2. The ice bath was removed and the reaction was stirred overnight, whereby TLC (EtOAc:hexane 2:1, developed with ninhydrin) showed that most of the acid was consumed. The pyridine was evaporated, and the white solid residue was washed with ether, filtered and dried. The product obtained was a white solid (18 g, 80% yield) and was used without further purification, m.p.: 236–238 °C. ^{1}H-NMR (acetone-d_6) δ 9.40 (brs; 1H, OH); 7.97 (d; $J = 9.0$ Hz, 2H, Ar-*H2*); 7.79 (d; $J = 9.0$ Hz, 2H, Ar–*H3*); 2.38 (t; $J = 7.5$ Hz, 2H, CH_2CO); 1.70 (sextet; $J = 7.5$ Hz, 2H, $MeCH_2$); 0.96 (t; $J = 7.5$ Hz, 3H, Me). ^{13}C-NMR (acetone-d_6) δ 172.39 (s; CONH); 167.28 (s; *C*OOH); 144.60 (s; Ar–*C4*); 131.44 (d; Ar–*C2* and Ar–*C6*); 125.68 (s; *C*OOH); 119.12 and 119.04 (d; Ar–*C3* and Ar–*C5*); 39.59 (t; CH_2CO); 19.40 (t; $MeCH_2$); 13.92 (q; Me). HRMS (DCI-CH_4) 208.0890 calc. 208.0974 (MH^+). Anal. $C_{11}H_{13}NO_3$ (C, H).

5.1.5. Pentanedioic acid monobutyryloxymethyl ester ***2a*** *and pentanedioic acid dibutyryloxymethyl ester* ***3a***

Et_3N (165 mL, 120 g, 1.2 mol) was added to a solution of chloromethyl butyrate (133.8 g, 0.98 mol) and glutaric acid (129 g, 0.98 mol) in acetone (500 mL). The mixture was refluxed for 12 h, whereupon a large amount of precipitate formed. The precipitate was filtered and washed with acetone. The filtrate was evaporated and the residue was partitioned with aqueous 1 M K_2CO_3 (500 mL) at a pH of about 8 and EtOAc (500 mL). The aqueous phase was extracted several times with EtOAc whilst maintaining the basic pH by continuous addition of solid K_2CO_3. The organic phase was washed (3×100 mL) with water at pH 8, and the washings were added to the aqueous phase. The combined aqueous phase was acidified with 2 M HCl and

was extracted with EtOAc (4×200 mL). The organic phase was collected, treated with charcoal, dried ($MgSO_4$) and evaporated to give the product **2a** as an oil, 110 g, 0.47 mol (48% yield) which was used without further purification. ^{1}H-NMR ($CDCl_3$) δ 5.76 (s; 2H, OCH_2O); 2.47 and 2.44 (two t; $J = 7.5$ Hz, 4H, $CH_2CH_2CH_2$); 2.35 (t; $J = 7.5$ Hz, 2H, $MeCH_2CH_2$); 1.98 (quintet; $J = 7.5$ Hz, 2H, $CH_2CH_2CH_2$); 1.67 (sextet; $J = 7.5$ Hz, 2H, $MeCH_2CH_2$); 0.95 (t; $J = 7.5$ Hz, 3H, Me). ^{13}C-NMR ($CDCl_3$) δ 178.72 (s; *C*OOH); 172.35 (s; $MeCH_2CH_2CO$); 171.61 (s; CH_2CO); 79.24 (t; OCH_2O); 35.83 (t; $MeCH_2CH_2$); 32.83 and 32.79 (two t; $CH_2CH_2CH_2$); 19,52 (t; $CH_2CH_2CH_2$); 18.14 (t; $MeCH_2CH_2$); 13.51 (q, Me). MS (CI–NH_3) 250 (MNH_4^+), 233 (MH^+). Anal. $C_{10}H_{16}O_6$ (C, H). The EtOAc extract from the basic aqueous solution was dried ($MgSO_4$) and evaporated to give an oily residue (76 g) containing traces of chloromethyl butyrate (as detected by NMR). The residue was kugelrohr distilled (190 °C/0.5Torr) to give 70 g, 0.20 mole (20% yield) of pure *bis*-ester **3a**. ^{1}H-NMR ($CDCl_3$) δ 5.74 (s; 4H, two OCH_2O); 2.43 (t; $J = 7.5$ Hz, 4H, $CH_2CH_2CH_2$); 2.33 (t; $J = 7.5$ Hz, 4H, two $MeCH_2CH_2$); 1.96 (quintet; $J = 7.5$ Hz, 2H, $CH_2CH_2CH_2$); 1.65 (sextet; $J = 7.5$ Hz, 4H, two $MeCH_2CH_2$); 0.94 (t, 6H, two Me).

*5.1.6. Pentanedioic acid mono-(1-butyryloxy-ethyl) ester **2b** and pentanedioic acid bis-(1-butyryloxy-ethyl) ester **3b***

Obtained in 36 and 25% yield, respectively, as described for **2a** and **3a**. **2b**, b.p. 165 °C/0.3 Torr (kugelrohr). ^{1}H-NMR ($CDCl_3$) δ 6.68 (q; $J = 5.5$ Hz, 1H, OC*H*O); 2.43 and 2.41 (two t; $J = 7.5$ Hz, 4H, $CH_2CH_2CH_2$); 2.29 (td; $J = 7.5$, 1.0 Hz, 2H, $MeCH_2CH_2$); 1.95 (quintet; $J = 7.5$ Hz, 2H, $CH_2CH_2CH_2$); 1.65 (sextet; $J = 7.5$ Hz, 2H, $MeCH_2CH_2$); 1.47 (d; $J = 5.5$ Hz, 3H, CH*Me*); 0.94 (t; $J = 7.5$ Hz, 3H, Me). ^{13}C-NMR ($CDCl_3$) δ 177.80 (s; *C*OOH); 170.69 (s; $MeCH_2CH_2CO$); 169.86 (s; CH_2CO); 87.48 (d; O*C*HO); 34.92 (t; $MeCH_2CH_2CO$); 31.94 and 31.76 (two t; $CH_2CH_2CH_2$); 18.52 (superimposed q and t; $CH_2CH_2CH_2$, OCH(*Me*)O), 17.13 (t; $MeCH_2CH_2$); 12.47 (q; Me). HRMS (CI–CH_4) 247.111000 (MH^+), calc. 247.154549, $C_{11}H_{18}O_6$. **3b**, b.p. 165–170 °C/0.3 Torr. ^{1}H-NMR ($CDCl_3$) δ 6.85 (q; $J = 5.5$ Hz, 1H, OC*H*O); 2.38 (t; $J = 7.5$ Hz, 4H, $CH_2CH_2CH_2$); 2.28 (td; $J = 7.5$, 1.0 Hz, 4H, two $MeCH_2CH_2$); 1.94 (quintet; $J = 7.5$ Hz, 2H, $CH_2CH_2CH_2$); 1.64 (sextet; $J = 7.5$ Hz, 4H, two $MeCH_2CH_2$); 1.46 (d; $J = 5.5$ Hz, 6H, two CH*Me*); 0.94 (t; $J = 7.5$ Hz, 6H, two Me). ^{13}C-NMR ($CDCl_3$) δ 171.54 (s; $MeCH_2CH_2CO$); 170.79 (s; CH_2CO); 88.38 (d; O*C*HO); 35.90 (t; $MeCH_2CH_2$); 32.82 (t; $CH_2CH_2CH_2$); 19.50 (superimposed q and t; $CH_2CH_2CH_2$, O_2CHMe); 18.11 (t; $MeCH_2CH_2$); 13.46 (q; Me).

*5.1.7. Butyric acid 2-(2-methoxy-ethoxy)-acetoxymethyl ester **8***

Chloromethyl butyrate (5 mL, 5.4 g 30.5 mmol) was added to a solution of 2-(2-methoxyethoxy)acetic acid (4.5 mL, 5.45 g 40.6 mmol) in acetone (30 mL), followed by the dropwise addition of Et_3N (6.6 mL, 1.2 equiv.). The mixture was heated at 40 °C for 20 h. The precipitate was filtered and washed with acetone. The filtrate was evaporated, and the residue (11 g) was washed thrice with water, thrice with 5% $NaHCO_3$ and thrice with brine, to give the crude product as a colorless oil, 4.6 g. The crude product was distilled at 140 °C/1 Torr, to give the product as an oil, 3.8 g (44% yield). ^{1}H-NMR ($CDCl_3$) δ 5.83 (s; 2H, OCH_2O); 4.30 (s; 2H, $COCH_2O$); 3.69 (m; 4H, $O(CH_2)_2O$); 3.43 (s; 3H, OMe); 2.42 (t; $J = 7.3$ Hz, 2H, $MeCH_2CH_2$); 1.62 (sextet; $J = 7.3$ Hz, 2H, $MeCH_2$); 0.90 (t; $J = 7.3$ Hz, 3H, Me). ^{13}C-NMR (D_2O) δ 175.72 (s; OCH_2CO); 171.62 (s; CH_2CH_2CO); 80.53 (t; OCH_2O); 71.53 (t; $MeOCH_2CH_2$); 70.83 (t; $MeOCH_2CH_2$); 68.15 (t; $COCH_2O$); 58.65 (q; *Me*O); 35.95 (t; CH_2CH_2CO); 18.26 (t; $MeCH_2$); 13.26 (q; Me). MS (CI–NH_3) 252 (MNH_4^+). Anal. $C_{10}H_{18}O_6$ (C, H).

*5.1.8. Butyric acid 2-[2-(2-methoxy-ethoxy)-ethoxy]-acetoxymethyl ester **9***

Obtained in 53% yield as described for **8**. ^{1}H-NMR ($CDCl_3$) δ 5.83 (s; 2H, OCH_2O); 4.30 (s; 2H, $COCH_2O$); 3.52–3.80 (m; 8H, $[O(CH_2)_2O]_2$); 3.38 (s; 3H, O*Me*); 2.43 (t; $J = 7.3$ Hz, 2H, CH_2CH_2CO); 1.62 (sextet; $J = 7.3$ Hz, 2H, $MeCH_2$); 0.97 (t; $J = 7.3$ Hz, 3H, Me). ^{13}C-NMR (D_2O) δ 175.61 (s; OCH_2CO); 171.57 (s; CH_2CH_2CO); 80.49 (t; OCH_2O); 71.50 (t; $MeOCH_2CH_2$); 70.94 (t; $COCH_2OCH_2CH_2$); 71.11 (t; $MeOCH_2CH_2$); 70.01 (t; $COCH_2OCH_2CH_2$); 68.13 (t; $COCH_2O$); 58.65 (q; *Me*O); 35.94 (t; $MeCH_2CH_2$); 18.27 (t; $MeCH_2CH_2$); 13.28 (q; Me). MS (CI–NH_3) 296 (MNH_4^+).

*5.1.9. Butyric acid diethoxy-phosphoryloxymethyl ester **10a***

Et_3N (22.2 mL, 1.2 equiv.) was added dropwise to a solution of diethyl phosphate (20.55 g, 0.133 mol) and

chloromethyl butyrate (18.62 g, 1 equiv.) in dry DMF (30 mL) under N_2. The reaction mixture was heated at 70 °C for 4 h, during which time a large amount of precipitate formed and TLC (EtOAc:hexane:*i*-PrOH 5:5:1, detection-vanillin) showed that most of the acid had reacted. The precipitate was filtered and washed with EtOAc. The filtrate was partitioned between water and EtOAc. The aqueous phase was washed with a small amount of EtOAc, and the combined organic phase was washed thrice with water, twice with 5% solution of $NaHCO_3$, twice with brine, dried ($MgSO_4$) and evaporated to give an oily crude product (25.5 g) which was distilled at 125 °C/0.1–0.2 Torr to give **10a** as a colorless oil (17.2 g, 50%). ^{1}H-NMR ($CDCl_3$) δ 5.63 (d; $J = 13$ Hz, 2H, OCH_2O); 4.13 (dquintets; $J = 7.0$, 0.8 Hz, 4H, two CH_2OP); 2.36 (t; $J = 7.5$ Hz, 2H, CH_2COO); 1.69 (sextet; $J = 7.5$ Hz, 2H, CH_2CH_2CO); 1.34 (td; $J = 7.0$, 1.1 Hz, 6H, two $MeCH_2OP$); 0.96 (t; $J = 7.5$ Hz, 3H, Me). ^{13}C-NMR ($CDCl_3$) δ 171.91 (s; CO), 82.40 (td; OCH_2O); 64.20 (td; $MeCH_2OP$); 35.78 (t; CH_2COO); 17.99 (t; CH_2CH_2CO); 15.50 (td; $MeCH_2OP$); 13.44 (q; Me). ^{31}P-NMR $CDCl_3$ δ −4.51. Anal. $C_9H_{19}O_6P{\cdot}0.5H_2O$ (C, H).

5.1.10. Butyric acid 1-(diethoxy-phosphoryloxy)-ethyl ester **10b**

Obtained in 62% yield as described for **10a**, b.p. 95–100 °C/0.3Torr. ^{1}H-NMR $CDCl_3$ δ 6.47 (dq; $J =$ 7.0, 5 Hz, 1H, OC*H*O); 4.08 (ddquintets; $J = 7.0$, 5.0, 0.8 Hz, 4H, two CH_2OP); 2.28 (t; $J = 7.5$ Hz, 2H, CH_2COO); 1.62 (sextet; $J = 7.5$ Hz, 2H, CH_2CH_2CO); 1.49 (d; $J = 5$ Hz, 3H, CH*Me*); 1.29 (tdd; $J = 7.0$, 1.5, 0.8 Hz, 6H, two $MeCH_2OP$); 0.91 (t; $J = 7.5$ Hz, 3H, Me). ^{13}C-NMR $CDCl_3$ δ 171.43 (s; CO); 91.07 (d; O*C*HO); 64.11 (td; CH_2OP); 35.99 (t; CH_2COO); 21.40 (qd; CH*Me*); 18.09 (t; CH_2CH_2CO); 16.06 (qd; $MeCH_2OP$); 13.55 (q; Me). HRMS (CI–CH_4), 267.0993 (M–H$^+$). Anal. $C_{10}H_{20}O_6P$ (C, H).

5.1.11. Nicotinic acid butyroyloxymethyl ester **4a**

Nicotinic acid (23.4 g, 190 mmol) was added to a solution of **1a** (26 g, 190 mmol) in DMF (90 mL) under N_2, followed by the dropwise addition of Et_3N (32 mL, 23.23 g, 1.2 equiv.) until the acid dissolved. The mixture was heated at 60 °C overnight, during which time a large amount of precipitate formed and TLC (EtOAc:hexane 2:1) showed that most of the acid was consumed. The precipitate was filtered and washed with EtOAc (300 mL). The solution was washed four times with water, the aqueous phase was extracted with a small amount of EtOAc (3×10 mL) and the combined organic phase was washed twice with brine, dried ($MgSO_4$) and evaporated. The product was obtained as a brownish oil (31.7 g), that was distilled at 109–110 °C (0.05 Torr) to give a colorless liquid (25 g, 59% yield) which was used without further purification. ^{1}H-NMR ($CDCl_3$) δ 9.26 (dd; $J = 2.0$, 1.0 Hz, 1H, Ar–H*2*); 8.82 (dd; $J = 5.0$, 2.0 Hz, 1H, Ar–H*6*); 8.34 (dt; $J = 8.0$, 2.0 Hz, 1H, Ar–H*4*); 7.43 (ddd; $J = 8.0$, 5.0, 1.0 Hz, 1H, Ar–H*5*); 6.04 (s; 2H, OCH_2O); 2.39 (t; $J = 7.5$ Hz, 2H, CH_2CO); 1.70 (sextet; $J = 7.5$ Hz, 2H, $MeCH_2$); 0.97 (t; $J = 7.5$ Hz, 3H, Me). ^{13}C-NMR ($CDCl_3$) δ 171.83 (s; CH_2CO); 163.68 (s; Py*C*O); 153.76 (d; Py–*C*2); 150.88 (d; Py–*C*6); 137.04 (d; Py–*C*4); 124.74 (s; Py–*C*3); 123.10 (d; Py–*C*5); 79.32 (t; OCH_2O); 35.35 (t; CH_2CO); 17.77 (t; $MeCH_2$); 13.17 (q; Me). HRMS (CI–CH_4) 224.0900 (MH$^+$), calc. 224.0923, $C_{11}H_{14}NO_4$.

5.1.12. Nicotinic acid 1-butyryloxy-ethyl ester **4b**

Obtained in 48% yield as described for **4a**, b.p. 135–140 °C/0.5 Torr. ^{1}H-NMR ($CDCl_3$) δ 9.20 (dd; $J = 2.0$, 1.0 Hz, 1H, Py–*H2*); 8.78 (dd; $J = 5.0$, 2.0 Hz, 1H, Py–*H6*); 8.28 (dt; $J = 8.0$, 2,0 Hz, 1H, Py–*H4*); 7.39 (ddd; $J = 8.0$, 5.0, 1.0 Hz, 1H, Py–*H5*); 7.12 (q; $J = 5.5$ Hz, 1H, OC*H*O); 2.32 (td; $J = 7.5$, 1.0 Hz, CH_2COO, 2H); 1.65 (sextet; $J = 7.5$ Hz, 2H, $MeCH_2$); 1.61 (d; $J = 5.5$ Hz, 3H, CH*Me*); 0.94 (t; $J = 7.5$ Hz, 3H, Me). ^{13}C-NMR ($CDCl_3$) δ 171.59 (s; CO–Bu); 163.31 (s; Py*C*O); 153.83 (d; Py–*C2*); 151.09 (d; Py–C6); 137.33 (d; Py–C4); 125.41 (s; Py–C3); 123.37 (d; Py–C5); 89.13 (d; OCHO); 35.92 (t; $COCH_2$), 19.65 (q; CHMe); 18.17 (t; $MeCH_2$); 13.52 (q; Me). HRMS (CI–CH_4) 238.1070 (MH$^+$), calc. 238.1079, $C_{12}H_{16}NO_4$.

5.1.13. 4-Amino-benzoic acid butyryloxymethyl ester **5**

4-Amino-benzoic acid (21.05 g, 153 mmol) was added to a solution of chloromethyl butyrate (21.5 g, 157 mmol) in DMF (60 mL) under N_2, followed by the dropwise addition of Et_3N (25.5 mL, 18.5 g, 1.2 equiv.) until the acid dissolved. The reaction mixture was heated at 60 °C overnight, during which time a large amount of precipitate formed and TLC (EtOAc:hexane 2:1) showed that most of the acid has reacted. The precipitate was filtered and washed with EtOAc. The solution was partitioned between water and EtOAc. The aqueous phase was extracted with a small amount of EtOAc, and the combined organic phase was washed thrice with a 5% solution of $NaHCO_3$, twice with water, twice with brine and dried ($MgSO_4$). Evaporation of the solvent gave a pasty residue that was triturated with

hexane to give 18 g of a white solid that was recrystallized from *i*-PrOH to give 15 g (41% yield) of product, m.p. 130–132 °C. ^{1}H-NMR ($CDCl_3$) δ 7.88 (d; $J = 9.0$ Hz, 2H, Ar–*H2*); 6.63 (d, $J = 9.0$ Hz, 2H, Ar–*H3*); 5.95 (s; 2H, OCH_2O); 4.75 (s; 2H, NH_2); 2.35 (t; $J = 7.5$ Hz, 2H, $COCH_2$); 1.67 (sextet; $J = 7.5$ Hz, 2H, $MeCH_2$); 0.94 (t; $J = 7.5$ Hz, 3H, Me). HRMS (CI–CH_4): 238.1080, calc. 238.1079 (MH^+) $C_{12}H_{16}NO_4$. In order to obtain the HCl salt, the product (pure or crude) was treated with 1 M HCl, in EtOAc (4 equiv.), and precipitated with dry ether. The precipitate was filtered, washed with ether and hexane. The crude salt was crystallized from CH_2Cl_2:ether to give the pure product, m.p. 133–135 °C. ^{1}H-NMR ($CDCl_3$) δ 7.56 (d; $J = 9.0$ Hz, 2H, Ar–*H2*); 6.63 (d; $J = 9.0$ Hz, 2H, Ar–*H3*); 5.95 (s; 2H, OCH_2O); 2.37 (t; $J = 7.5$ Hz, 2H, CH_2CO); 1.67 (sextet; $J = 7.5$ Hz, 2H, $MeCH_2$); 0.94 (t; $J = 7.5$ Hz, 3H, Me).

5.1.14. 4-Butyramino-benzoic acid butyryloxymethyl ester **7a**

Obtained in 77% yield as described for **4a**, m.p. 89–90 °C. ^{1}H-NMR ($CDCl_3$) δ 8.03 (d; $J = 8.0$ Hz, 2H, Ar-H2); 7.62 (d; $J = 8.0$ Hz, 2H, Ar–H3); 5.98 (s; 2H, OCH_2O); 2.37, 2.36 (two t; $J = 7.5$ Hz, 4H, $OCOCH_2$); 1.76 (sextet; $J = 7.5$ Hz, 2H, CH_2 CH_2CON); 1.67 (sextet; $J = 7.5$ Hz, 2H, $MeCH_2CH_2COO$); 1.01 and 0.94 (two t; $J = 7.5$ Hz, 6H, two Me). ^{13}C-NMR ($CDCl_3$) δ 172.47 (s; *C*ONH); 171.52 (s; *C*O-acetal); 164.70 (s; Ar–*C*O); 142.85 (s; Ar–*C4*); 131.35 (d; Ar–*C2* and Ar–*C6*); 124.12 (s; Ar–*C1*); 118.73 (d; Ar–*C3* and Ar–*C5*); 79.42 (t; OCH_2O); 39.71 (t; $NCOCH_2$); 35.83 (t; $OCOCH_2$); 18.85, 18.11 (two t; two $MeCH_2CH_2CO$); 13.70 and 13.49 (two q; two Me). HRMS (DCI–CH_4) 308.1480, calc. 308.1498 (MH^+) $C_{16}H_{22}NO_5$. Anal. $C_{16}H_{21}NO_5$ (C, H, N).

5.1.15. 4-Butyramino-benzoic acid 1-butyryloxy-ethyl ester **7b**

Obtained in 14% yield as described for **4a**, m.p. 65–66 °C. ^{1}H-NMR ($CDCl_3$) δ 7.98 (d; $J = 8.0$ Hz, 2H, Ar–*H2*); 7.61 (d; $J = 8.0$ Hz, 2H, Ar–*H3*); 7.45 (brs; 1H, NH); 7.10 (q; $J = 5.5$ Hz, 1H, OC*H*O); 2.37 (t; $J = 7.5$ Hz, 2H, $NHCOCH_2$); 2.32, 2.31 (two t; $J = 7.5$ Hz, 2H, $OCOCH_2$); 1.76 (sextet; $J = 7.5$ Hz, 2H, CH_2CH_2CON); 1.66 (sextet; $J = 7.5$ Hz, 2H, CH_2CH_2COO); 1.59 (d; $J = 5.5$ Hz, 3H, CH*Me*); 1.00, 0.94 (two t; $J = 7.5$ Hz, 6H, two Me). ^{13}C-NMR ($CDCl_3$) δ 171.73 (s; *C*ONH, *C*O-acetal); 164.01 (s; Ar–*C*O); 142.72 (s; Ar–*C*NH); 131.08 (d; Ar–*C2* and Ar–*C6*); 124.40 (s; Ar–*C*O); 118.70 (d; Ar–*C3* and Ar–*C5*); 88.79 (d; O*C*HO); 39.62 (t; $NCOCH_2$); 35.95 (t; $OCOCH_2$); 19.58 (t; O*C*HO); 18.82, 18.15 (two t; two CH_2CH_2CO); 13.67, 13.46 (two q; two Me). HRMS (DCI–CH_4) 322.1640, calc. 322.1654 (MH^+) $C_{17}H_{24}NO_5$.

5.2. Biology

5.2.1. Materials

RPMI-1640 medium, fetal calf serum (FCS) and all other tissue culture materials were obtained from Biological Industries Beth-Haemek, Israel; ^{3}[H]-thymidine from ICN, England; and other reagents from Sigma.

5.2.2. Cells

HT-29, MCF-7, $MIAPaCa_2$, PC-3, LnCap, HL-60 and K-562 cells were acquired from ATCC. They were grown in RPMI-1640 medium supplemented with 10% FCS, penicillin (250 U mL^{-1}), and streptomycin (125 μg mL^{-1}). The cells were transferred into fresh medium twice weekly and were incubated at 37 °C in a humidified 5% CO_2 incubator.

5.2.3. Treatment with prodrugs

The lipophilic prodrugs were solubilized in DMSO. The concentrated DMSO solutions were diluted with medium so that the highest concentration of DMSO in the test medium was 0.1%. Solutions of the prodrugs were handled using Hamilton syringes and Teflon or glass vials. In parallel to the test compounds, appropriate controls were run using 0.1% DMSO in medium.

5.2.4. Proliferation assays

Cells, 100–200 μL, at a density of $2–5\times10^4$ cells mL^{-1} were seeded in tissue culture 96-well plates (in triplicate) for 24 h. They were then exposed to different concentrations of the prodrugs for the specified times. Three methods were used for proliferation measurements: a) *SRB assay*. A modified procedure of Papazisis et al. [27], was employed for HT-29, MCF-7 and $MIAPaCa_2$ cell lines. Briefly, after 7 days of cell incubation with the test compounds the samples were fixed by the addition of 50% trichloroacetic acid at 4 °C and stained with 0.4% SRB in 1% acetic acid. The samples were then rinsed with 1% acetic acid, air-dried and the SRB dye was solubilized in 10 mM TRIS, pH 10, and absorbance was read at 570 nm. b) *XTT proliferation assay*. This assay was used with LnCap and PC-3 human prostate cancer cells. The cells were exposed to the test compound for 48 h and analyzed as described [28].

c) *Thymidine uptake*. HL-60 and K562 cells at 1×10^5 mL^{-1} were treated with the prodrugs for 3 days. Aliquots of 150 μL were transferred into 96-well plates, and 20 μL of ^{3}[H]-thymidine, 6.7 Ci $mmole^{-1}$ specific activity (final radioactivity 5 μCi mL^{-1}) was added. After 16 h, the cells were washed and harvested (PHD, Cambridge Technology, Inc., Cambridge, MA). The filters were dried and 2 mL scintillation fluid added. Radioactivity was measured for 5 sec/sample in a 1217 RackBeta (LKB, Sweden) counter. Background counts were deducted from the radioactivity of the cell samples. The mean value obtained from 4 wells was computed by the program.

5.2.5. Induction of differentiation

Cancer cell differentiation was evaluated with the human myeloid leukemic cell line HL-60 by NBT reduction activity [1, 2]. Cell cultures, after 3 days of exposure to the test compounds, were stimulated with 0.4 μM 12-O-tetradecanoyl-phorbol 13-acetate (PMA) in the presence of 0.1% NBT. After 30 min of incubation at 37 °C they were examined microscopically by scoring at least 200 cells. The % of NBT positive cells was calculated from the ratio the stained cells to the total cells scored and the % of positive cells in untreated culture was subtracted. NBT reduction activity correlated with morphological changes observed with May–Grunwald Giemsa stained cytospin smears of the cells.

5.2.6. Apoptosis

The cells were exposed to the drugs for 3 days, harvested, washed in PBS and resuspended in binding buffer (20 mM HEPES, pH 7.4, 150 mM NaCl, 2.5 mM $CaCl_2$). FITC-conjugated annexin (1:100 dilution of 50 μg mL^{-1}) and 1:100 dilution of propidium iodide (0.5 mg mL^{-1} PBS) were added. After 10 min on ice, the cells were analyzed by flow cytometry. Labeled cells were run in a FACStarplus flow cytometer (Becton-Dickinson, Immunofluorometry systems, Mountain View, CA). Cells, negative for propidium iodide and positive for annexin, were regarded as apoptotic cells [29].

5.2.7. Quantitative measurement of Hb

K562 cells were cultured at 2×10^5 cells mL^{-1}, 3 mL/well, harvested on day 3, counted, lyzed in water and Hb was determined by ion-exchange HPLC as described [31].

5.2.8. Toxicity studies in mice

Compounds **2a**, **2b** or **10a** were dissolved in a solution of 20 mM acetate buffer, pH 6, and 10% DMSO to give a final concentration of 20 mg mL^{-1}. The **2a** lysine salt was dissolved in 20 mM acetate buffer, pH 6, to give a final concentration of 30 mg mL^{-1}. Male Balb-c mice, 8–10 weeks old, were obtained from Bar-Ilan University animal colony. The formulated compounds were injected i.p. into the mice at a rate of 0.4 mL min^{-1}. Each animal was weighed and adjustments of dose and volume were made. All the experiments with mice were conducted following the NIH laboratory animal care guidelines and in accordance with the guidelines of Beilinson Campus Helsinki committee for animal care.

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